Enzyme Levels in Raw Meat After Low Dose Ionizing Radiation and Extended Refrigerated Storage

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ABSTRACT

Bovine semimembranosus *muscle samples obtained aseptically 24 h after slaughter were irradiated at 1, 5 and 10 kGy between 0 and 4°C and stored at 2°C to determine effects of low level ionizing radiation on tissue-free enzyme levels. Samples were analyzed after 1, 7, 14 and 28 days of storage. Nonspecific proteolysis was measured using ^{14}C-hemoglobin as the substrate. For determination of possible effect by irradiation on lysosomal enzymes, acid phosphatase and β-glucuronidase were measured. Assays for acid phosphatase and hemoglobin proteolysis indicated negative relationship between increasing dose and enzymatic activity. At 10 kGy mean reductions in enzymatic activity were 8% and 42%, respectively. β-Glucuronidase was not affected at this or at the lower levels of radiation. Correlation between dosage and storage time with enzymatic activity was not significant.*

INTRODUCTION

Raw meat normally cannot be stored for extended periods under refrigeration (0–4°C) because of proliferation of psychotropic food spoilage microorganisms and texture degradation caused by the presence of endogenous proteolytic enzymes. Subjecting raw meat to 4 kGy of ionizing radiation can reduce or eliminate many food spoilage microorganisms, but radiation is not especially effective in substantially reducing proteolysis.

Meat Science 0309-1740/88/$03·50 © 1988 Elsevier Science Publishers Ltd, England. Printed in Great Britain

Doty & Wachter (1955), by measuring liberated tyrosine, estimated that proteinase activity was reduced by 50% when beef was irradiated with 13 kGy. Irradiation of beef at 45 kGy (4°C) destroyed approximately 75% of proteolytic activity (Losty *et al.*, 1973). Studies on tissue homogenates and model systems by Rhodes & Meegunwan (1962) demonstrated that high dose irradiation caused only partial deactivation of proteolytic enzymes; complete deactivation did not occur even at 400 kGy. Total (95–100%) inactivation of proteolytic enzymes required that meat be blanched at 70°C for 5 min prior to irradiation (Losty *et al.*, 1973). Organoleptic studies conducted by Coleby *et al.* (1961) on beef and pork irradiated at 20 kGy (room temperature) or 50 kGy (−75°C), however, revealed an immediate softening of tissue and further breakdown during storage.

Numerous investigations, particularly during the 1960s, were conducted to study the direct effects of ionizing radiation on enzymes. Bier & Nord (1952) ascertained that pure enzymes were more stable in the dry state. Siu (1957) in a review article enumerated the conditions which significantly influenced enzymatic activities: pH, enzyme concentration, physical state of the enzyme, temperature during irradiation, presence of other compounds, and condition of the cells—intact versus homogenate. These, with other findings by Harris (1970), indicate that enzyme activity is sensitive to, and is affected to a large degree by, its immediate environment.

In the United States, the use of low dosage ionizing radiation has been approved to eradicate trichina in pork; its use to reduce microbial flora in chicken is presently under consideration. The effect of low dosage ionizing radiation on the status of proteolytic enzymes in intact muscle beef under conditions (temperature, storage time) that approximate those which might be used during commercial conditions should be reexamined to determine the relationship between radiation and protease activity more closely. In this study we measured the enzyme activity in bovine muscle after low dose (1–10 kGy) irradiation and after subsequent storage at 2°C for up to 28 days. The samples were prepared under aseptic conditions to exclude interference by exogenous microbial proteolytic activity both in the controls and in those samples receiving only minimal radiation.

MATERIALS AND METHODS

Processing

Bovine *semimembranosus*, obtained 24 h post mortem, was handled in a manner which minimized microbial contamination. All surfaces and knives to come in contact with the meat were sterilized, and the outer portion of the

meat was removed to expose a sterile inner core. Samples (3 × 3 × 2 in) were wrapped in tissue paper premoistened with an aqueous antibiotic solution (20 mg neomycin and 10 mg streptomycin/100 ml) and placed in hermetically sealed evacuated plastic pouches (International Kenfield IKD All Vac No. 13),* having low oxygen permeability (1·0 cc/100 in^2/24 h). The samples were irradiated with a ^{137}Cs source (dose rate 0·13 kGy/min) to levels of 1, 5, 10 kGy. The temperature of the samples was maintained between 0–4°C during irradiation. Samples were stored at 2°C for 1, 7, 14 and 28 days.

Prior to enzyme analyses, the outer surfaces of the muscle previously in contact with the antibiotics were trimmed, and tissue was excised in order to determine total microbial plate counts (aerobic and anaerobic). All visible connective and adipose tissue was removed. Samples were ground through a sterilized 3 mm plate. Ten grams of ground tissue was placed into 25 ml of a 0·25M sucrose solution containing 0·02M KCl, cooled in an ice bath, and homogenized with a Virtis '23' blender for 30 s (Moeller *et al.*, 1976). The homogenate was filtered through premoistened cheese cloth to remove connective tissue. The filtrate was centrifuged at 700 × *g* in a refrigerated centrifuge (0–4°C) for 10 min to yield a precipitate containing a nuclear fraction and a supernatant fraction containing lysosomes, mitochondria and microsomes as well as soluble components.

Enzyme assay

General protease activity was measured with ^{14}C hemoglobin as the substrate. The labelled substrate was prepared from bovine hemoglobin (Sigma Chem. Co.) and $K^{14}CNO$ (50 mCi/mmol—New England Nuclear) using the procedure described by Roth *et al.* (1971). To a microcentrifuge tube were added 0·6 ml sample, 0·7 ml of 0·2M acetate buffer pH 3·8, 0·1 ml antibiotic solution, and 0·2 ml ^{14}C hemoglobin (200 000 dpm). The samples were incubated at 37°C with continuous agitation for 24 h. A 0·85 ml aliquot was removed and the reaction was terminated by the addition of 0·2 ml of 50% trichloroacetic acid. The tubes were centrifuged and the supernatant removed and filtered through glass wool. Filtrate (0·1 ml) was added to a vial containing 7 ml Ecoscint (a scintillation fluor [National Diagnostics]) and counted in Beckman scintillation counter, model LS 8100. Enzymatic activity was determined after subtraction of the sample and reagent blanks.

Acid phosphatase activity was determined spectrophotometrically on the samples with *p*-nitrophenyl phosphate, disodium salt as the substrate. A 50 μl sample was incubated in a test tube in a shaking water bath at 37°C for 30 min at pH 4·8 with 0·5 ml citrate buffer (90 mmol) and 0·5 ml substrate.

* Reference to brand or firm name does not constitute endorsement by the US Department of Agriculture over others of a similar nature not mentioned.

Sample blanks contained 6·0 ml 0·1 N NaOH and 50 μl sample. Reaction was terminated by the addition of 5·0 ml of 0·1 N NaOH. Absorbance was measured on a spectrophotometer at 410 nm. Reagent and sample blanks were duly subtracted.

β-Glucuronidase activity of the samples was measured fluorometrically (Moeller *et al.*, 1976) with 0·5 mM 4-methylumbelliferyl-β-D-glucuronide (Sigma Chem. Co.), the substrate was dissolved in 0·25 M sucrose containing 0·1 M citrate buffer (pH 5·0). To 0·1 ml sample was added 0·4 ml citrate buffer and 1·5 ml substrate. The samples, sample blanks, and reagent blanks, were incubated at 37°C for 20 min. The reaction was stopped by the addition of 2·0 ml 1·0 M Na_2CO_3. The samples were centrifuged, and the fluorescence was measured on the clarified solution with a spectrophotofluorometer set at: excitation 360 nm, emission 448 nm.

To determine the extent that the enzymes present in the supernatant fraction were in the bound form, several samples were subjected to repeated freeze–thaw cycles (5–10) or treated with 0·01% Triton X.

The Biuret procedure described by Gornall *et al.* (1949) was used to determine protein concentration.

Microbiology

Aerobic media

Brain heart infusion (Difco) plus 2% agar (Difco) were dissolved in 500 ml water and sterilized by autoclaving. A sterile solution containing a thiamine supplement (5 μg/ml) was then added to the media. Samples were prepared in Stomacher '400' bags (25 gm meat/100 ml sterile peptone solution). Surface-plated samples were incubated at 20°C for 48 h.

Anaerobic media

Assay media was prepared as described by Dymicky *et al.* (1987). Media were autoclaved. Pour plated samples were sealed in anaerobic Oxoid jars, flushed with nitrogen, and incubated at 37°C for 48 h.

RESULTS AND DISCUSSION

The effect of gamma radiation on the free enzyme activity in raw muscle was determined initially in both a supernatant containing the lysosomes, mitochondria, microsomes, and soluble components, as well as in a precipitate containing the nuclear fraction. While the specific activities of the precipitate and supernatant fraction were roughly equivalent, approximately 80% of the total activity resided in the supernatant fraction

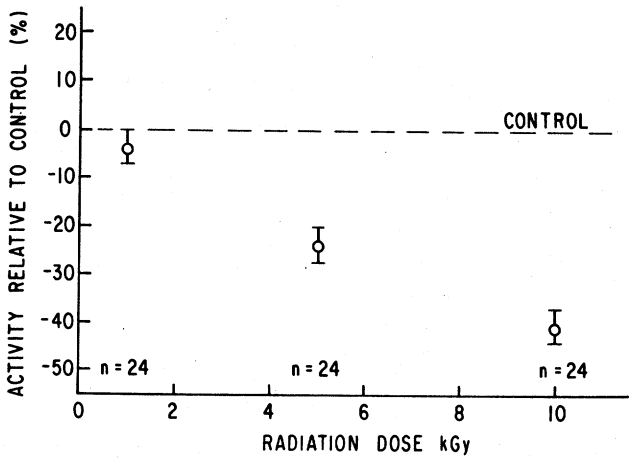

Fig. 1. Relationship between radiation dose and proteolytic activity as determined on a ^{14}C hemoglobin substrate relative to unirradiated control. Error bars—mean and 95% confidence limits.

containing lysosomal and non-lysosomal hydrolases. Lysosomes have been shown to contain more than 50 powerful hydrolases which are capable of degrading proteins, lipids, and carbohydrates (de Duve, 1983). All analyses reported in this study were conducted on this supernatant fraction. Enzymes of lysosomal origin measured included acid phosphatase and β-glucuronidase. General protease activity was measured using hemoglobin as the substrate.

^{14}C Hemoglobin substrate

Hemoglobin was used as a substrate by Anson (1938) to measure proteinase activity. The addition of a ^{14}C label to the hemoglobin by Losty *et al.* (1973) made it possible to measure extremely low levels of proteolytic activities not previously possible using spectrophotometric procedures. Proteolysis of hemoglobin results in the release of radioactive amino acids.

The relative change in proteolytic activity due to irradiation at 1, 5, 10 kGy compared to the activity of the unirradiated controls is presented in Fig. 1. Hemoglobin was the substrate. At 1 kGy there was a mean decrease of 4% relative to the control. At 5 and 10 kGy the decrease in activity was 24% and 41%, respectively. The analysis of variance (ANOVA) and regression analysis of the data indicate statistically significant logarithmic relation between dose and free enzyme activity. The following equation decribes the system:

$$\log_e [\text{per cent change} + 70] = -9{\cdot}76 \times 10^{-3} \times \text{dose} + 4{\cdot}26$$
$$r = -0{\cdot}745 \qquad p > 0{\cdot}01$$

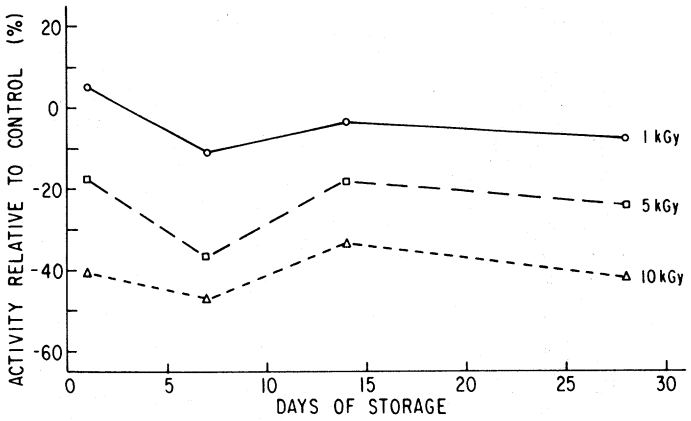

Fig. 2. Proteolytic activity (^{14}C hemoglobin substrate) as affected by ^{137}Cs radiation and storage at 2°C; relative to unirradiated control. $n = 6$. Pooled standard error = 5·18.

The relationship between enzymatic activity and storage time for different levels of ionizing radiation dosage is presented in Fig. 2. On the first day of storage, the proteolytic activity in the samples irradiated with 1 kGy was 6% greater than the control. Also evident was the inverse relation between dose (1, 5, 10 kGy) and proteolytic activity. Regression analysis of the data indicated that there was no statistically significant correlation ($r = -0{\cdot}032$; $p > 0{\cdot}05$) between duration of storage, level of irradiation of aseptically prepared tissue, and enzymatic activity. The enzymatic activity of the samples remained relatively constant during refrigerated storage.

Acid phosphatase

To determine the effect of radiation on the enzymes of lysosomal origin, and by inference obtain possible information on a large number of hydrolytic enzymes, we conducted assays for acid phosphatase and β-glucuronidase. Acid phosphatase activity was measured using *p*-nitrophenyl phosphate as the substrate, since high levels of inorganic phosphate in tissue precluded the use of β-glycerolphosphate. The acid phosphatase activity relative to the control after 1 kGy, 5 kGy, 10 kGy of radiation is presented in Fig. 3. The administration of 1 kGy of radiation caused no apparent change in enzymatic activity. Decreases in acid phosphatase activity of 5% and 8% were detected after irradiation with 5 kGy and 10 kGy, respectively. Regression analysis indicated a statistically significant logarithmic relation between dose and enzyme activity. The following equation describes the system:

$$\log_e[\text{per cent change} + 30] = -4{\cdot}06 \times 10^{-4} \times \text{dose} + 3{\cdot}38$$
$$r = -0{\cdot}33, \qquad p > 0{\cdot}01$$

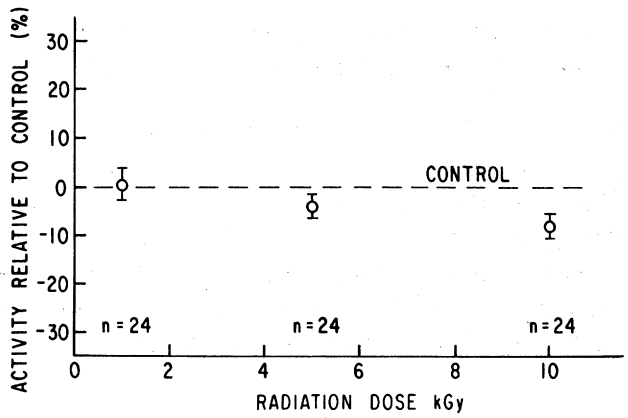

Fig. 3. Relationship between radiation dose and acid phosphatase activity relative to unirradiated control. Error bars—mean and 95% confidence limits.

Relative acid phosphatase activities of irradiated and unirradiated bovine muscle stored for periods of up to 28 days are presented in Fig. 4. Analysis of the data indicate no statistically significant correlation between dosage and length of storage ($r = 0{\cdot}045$; $p > 0{\cdot}05$). It should be noted that samples irradiated with 10 kGy showed a consistent decrease in activity with length of storage. Samples irradiated at 1 and 5 kGy initially demonstrated a decrease in activity during the first half of the 28-day period, followed by an increase in enzymatic activity. Some of the increased activity may result from hydrolysis of *p*-nitrophenylphosphate by microsomal glucose-6-phosphatase. Kas *et al.* (1983) reported that microsomal membranes lose their integrity approximately 9 or more days post mortem. This may account

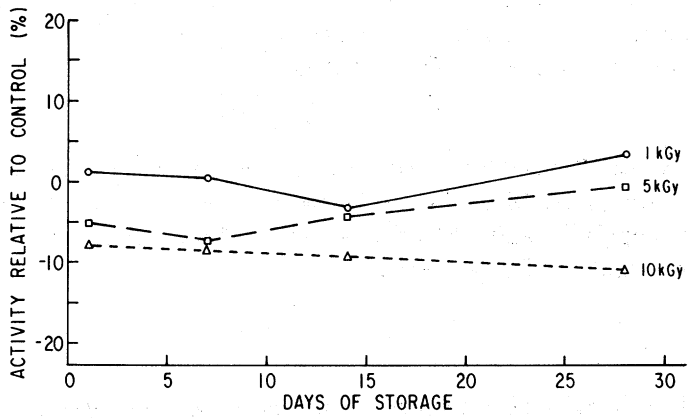

Fig. 4. Acid phosphatase activity relative to unirradiated control, after ^{137}Cs irradiation and storage at 2°C. $n = 6$. Pooled standard error = 2·45.

for some of the increased activity at 1 and 5 kGy. Radiation greater than 5 kGy may be sufficient to inactivate glucose-6-phosphatase.

β-Glucuronidase

β-Glucuronidase, another lysosomal enzyme, was measured using 4-methylumbelliferyl-β-D-glucuronide as the substrate. Activity was determined by measuring the resulting fluorescent product. β-Glucuronidase activity was determined only on two out of the seven sets of meat samples which were studied over the 28-day periods. Figure 5 is a plot of the effect of

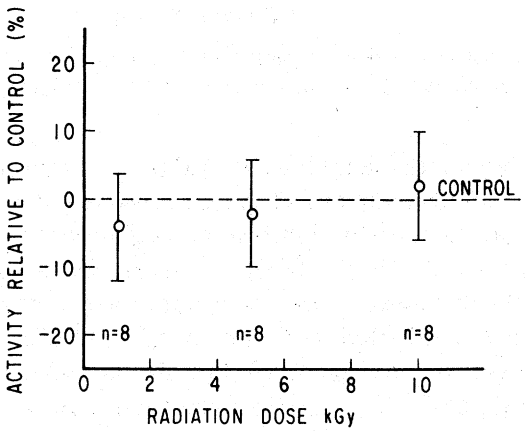

Fig. 5. Relationship between radiation dose and β-glucuronidase activity relative to unirradiated control. Errors bars—mean and 95% confidence limits.

radiation at 1, 5, 10 kGy on β-glucuronidase activity relative to the unirradiated control. From the limited data, it appears that the log activity of this enzyme is not affected by radiation.

$$r = 0{\cdot}202 \qquad p > 0{\cdot}05$$

A study by Gore *et al.* (1982) conducted on four species of fish which were irradiated, concluded that β-glucuronidase and four other lysosomal enzymes (cathepsin D, acid ribonuclease, aryl sulfatase, and β-galactosidase) were also not affected by radiation.

pH

The pHs of the irradiated and unirradiated meat samples were unaffected by either ionizing radiation or storage. pH values ranged from 5·6 to 6·0.

Aerobic and anaerobic plate counts on the muscle tissue indicated that the sterile techniques employed in the preparation of the samples and the use of antibiotics were effective in preventing or reducing microbial contamination in the controls and the samples which received sublethal doses of radiation. Plate counts averaged fewer than 100 bacteria per gram of tissue. The observed changes in enzymatic activity were therefore not due to microbial contamination.

CONCLUSION

Low level ionizing radiation above 1 kGy can reduce the activity of some proteolytic enzymes in muscle. The decrease in activity is dose and enzyme dependent, some enzymes being more sensitive to radiation than others. At 10 kGy, the maximum level tested, the enzymatic activity of β-glucuronidase was not affected, the acid phosphatase activity reduced by 8%, and general proteolytic activity reduced by 42% when measured using ^{14}C hemoglobin as the substrate. The enzyme levels in the tissue after irradiation and upon subsequent refrigeration at 2°C appeared to be independent of duration of storage (for up to 28 days) when compared to their sterile controls.

The results indicate that irradiation of meat between 1–10 kGy may be beneficial, by reducing proteolysis caused by some endogenous enzymes. After microbial spoilage, proteolysis is the major cause of deterioration in foods stored for extended periods, with greater breakdown occurring in organ tissue than in muscle. It is known that radiation above 4 kGy will reduce or eliminate most food spoilage microorganisms. With proper handling, low dose irradiation could be effective in short term extension of the shelf life of fresh meat without significant textural deterioration and may facilitate additional centralized meat packaging. However, irradiation cannot indefinitely extend the shelf life of fresh meat.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the help of Ron Jenkins, Robert Gates, Linda Garzarella, Christopher Thomas, and John Phillips for the statistical evaluation.

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